



TITLE:

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Taxonomic Relationship of Two Newt Species of *Pachytriton* Recently Described from China (Amphibia: Urodela: Salamandridae)

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Abstract: We assessed the taxonomic relationship of *Pachytriton changi* Nishikawa, Matsui et Jiang, 2012 and *P. xanthopilos* Wu, Wang et Hanken, 2012 using partial mitochondrial DNA sequence and found them to be so closely related as to be regarded as conspecific. This result, together with available morphological information, strongly indicates that *P. xanthopilos* is a subjective junior synonym of *P. changi*. By this synonymization, southern Hunan and northern Guangdong provinces are shown to be a part of the natural distribution range of *P. changi*, whose type locality is known only as China. However, known localities in this range are restricted and endangered status of the species is not changed.

Key words: Specific status; Synonymy; *Pachytriton*; Molecular phylogeny; China

INTRODUCTION

The salamandrid genus *Pachytriton* Boulenger, 1878, occurs in eastern and southeastern China, and currently consists of eight nominal species, *Pac. archospotus* Shen, Shen et Mo, 2008, *Pac. brevipes* (Sauvage, 1876), *Pac. changi* Nishikawa, Matsui et Jiang, 2012, *Pac. feii* Nishikawa, Jiang et Matsui, 2011, *Pac. granulosus* Chang, 1933, *Pac. inexpectatus* Nishikawa, Jiang, Matsui et Mo, 2011, *Pac. moi* Nishikawa, Jiang et Matsui, 2011, and *Pac. xanthopilos* Wu, Wang et Hanken, 2012 (after Wu et al., 2012). Among them, *Pac.*

xanthopilos was most recently described from the Mt. Mang in the Nanling Mountain Range, Yizhang County, Hunan Province (Wu et al., 2012). However, the species looks superficially very similar to *Pac. changi*, which was described based on two individuals sold at pet shops in Japan (Nishikawa et al., 2012). According to the original description and our examination, *Pac. xanthopilos* shares similar morphological characteristics such as large body, uniformly brown to light brown dorsal color, and orange spots along the flanks, with *Pac. changi*. In order to examine the taxonomic relationship between *Pac. xanthopilos* and *Pac. changi*, we compared the two species by use of mitochondrial DNA sequencing and external morphology.

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MATERIALS AND METHODS

We obtained sequence data of cytochrome b region (cyt b) of mitochondrial DNA (mtDNA) from muscle or liver tissue samples preserved in 99% ethanol. Methods for DNA extraction and, amplification and sequencing of the DNA fragment are same as those reported by Yoshikawa et al. (2008) with a modification for primers. To amplify the entire cyt b gene, we used the PCR primers HYD_Cytb_F1 (forward: 5'-CYAAYCCTAAAGCWGCAAAATA-3'; Matsui et al., 2008), Pachy_Cytb_530F (forward: 5'-CTGTAGACAAGRCTACCYTGACACG-3'; Nishikawa et al., 2011a), H14778 (reverse: 5'-AARTAYGGGTGRAADGRRAYTTTRTCT-3'; Matsui et al., 2007), and Salamander_Cytb_RN2 (reverse: 5'-YTYTCAATCTTKGGYTTACAA GACC-3'; Matsui et al., 2008). Sequences of complete cyt b gene have proved useful for delineating species in *Pachytriton* and related genera (Wu et al., 2010, 2012; Nishikawa et al., 2011a; Tominaga et al., 2013).

We reconstructed phylogenetic trees from the complete sequence of cyt b using our own samples of 15 specimens of *Pachytriton* and single representatives of *Cynops orientalis* and *Paramesotriton chinensis* (present study and Nishikawa et al., 2011a). For comparisons, published DNA sequence data for three type specimens of *Pachytriton xanthospilos* (Wu et al., 2012) and a single specimen of *Par. hongkongensis* (Zhang et al., 2005) were obtained from GenBank and added to our analyses (Table 1). Outgroup taxa (*Cynops* and *Paramesotriton*) were selected based on the results of Chan et al. (2001) and Weisrock et al. (2006).

The optimum substitution models for each codon position (1st, 2nd, and 3rd positions) were selected by Kakusan4 (Tanabe, 2011). We then constructed phylogenetic trees by maximum likelihood (ML) and Bayesian inference (BI) methods. The ML tree was sought using TREEFINDER ver. Mar. 2011 (Jobb, 2011) and Phylogears2 (Tanabe, 2008) with

TABLE 1. Species studied, with localities, GenBank accession numbers of sequence data, source, and information on voucher specimens. CIB: Chengdu Institute of Biology; KUHE: Graduate School of Human and Environmental Studies, Kyoto University.

No.	Species	Sampling locality	GenBank no.	Source	Vouchers	Types
1	<i>Pachytriton archospotus</i>	Guidong, Hunan	AB812098	This study	CIB GD2009091913	Topotype
2	<i>Pac. brevipes</i>	Zixi, Jiangxi	AB638656	Nishikawa et al. (2011a)	CIB JX200806238	Topotype
3	<i>Pac. changi</i>	Unknown locality, China	AB638711	Nishikawa et al. (2011a)	KUHE 39832	Holotype
4	<i>Pac. changi</i>	Unknown locality, China	AB638709	Nishikawa et al. (2011a)	KUHE 39763	Paratype
5	<i>Pac. feii</i>	Huangshan, Anhui	AB638629	Nishikawa et al. (2011a)	CIB 200805012	Holotype
6	<i>Pac. granulosus</i>	Tiantai, Zhejiang	AB638633	Nishikawa et al. (2011a)	CIB ZJ200806006	Topotype
7	<i>Pac. inexpectatus</i>	Jinxu, Guangxi	AB638707	Nishikawa et al. (2011a)	CIB GX20071101	Paratype
8	<i>Pac. moi</i>	Longsheng, Guangxi	AB638697	Nishikawa et al. (2011a)	CIB GX20070009	Holotype
9	<i>Pac. xanthospilos</i>	Yizhang, Hunan	JX237762	Wu et al. (2012)	CIB 97902	Holotype
10	<i>Pac. xanthospilos</i>	Yizhang, Hunan	JX237763	Wu et al. (2012)	CIB 97903	Paratype
11	<i>Pac. xanthospilos</i>	Yizhang, Hunan	JX237764	Wu et al. (2012)	CIB 97904	Paratype
12	<i>Pac. xanthospilos</i>	Yizhang, Hunan	AB812099	This study	CIB HN201108137	Topotype
13	<i>Pac. xanthospilos</i>	Yizhang, Hunan	AB812100	This study	CIB HN201108138	Topotype
14	<i>Pac. xanthospilos</i>	Shaoguan, Guangdong	AB812101	This study	CIB GD201108042	
15	<i>Pac. xanthospilos</i>	Mt. Mang, China (pet-traded)	AB812102	This study	KUHE 44985	
16	<i>Pac. xanthospilos</i>	Mt. Mang, China (pet-traded)	AB812103	This study	KUHE 44986	
17	<i>Pac. xanthospilos</i>	Mt. Mang, China (pet-traded)	AB812104	This study	KUHE 44987	
18	<i>Pac. xanthospilos</i>	Mt. Mang, China (pet-traded)	AB812105	This study	KUHE 44988	
19	<i>Paramesotriton chinensis</i>	Tiantai, Zhejiang	AB638712	Nishikawa et al. (2011a)	CIB ZJ200806081	
20	<i>Par. hongkongensis</i>	Unknown locality, Hongkong	AY458597	Zhang et al. (2005)		Topotype
21	<i>Cynops orientalis</i>	Jiangshan, Zhejiang	AB638713	Nishikawa et al. (2011a)	CIB ZJ200806473	

1000 trials of the likelihood ratchet method (Vos, 2003), and the Bayesian analysis was conducted with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001).

For the ML analysis, the J2 model (Jobb, 2011) with a gamma shape parameter (G), the TrN model (Tamura and Nei, 1993), and J2+G were selected by Kakusan4 as the optimal models for the 1st, 2nd, and 3rd codon positions, respectively. For the Bayesian analyses, the K80 (Kimura, 1980)+G, HKY (Hasegawa et al., 1985), and the GTR (Tavaré, 1986)+G were selected as the best substitution model for the 1st, 2nd, and 3rd codon positions, respectively. Two independent runs of four Markov chains were conducted for 20 million generations. We sampled one tree every 100 generations and calculated a consensus topology for 150,000 trees after discarding the first 50,001 trees (burn-in=5,000,000 generations).

The robustness of the ML tree was tested using bootstrap analysis (Felsenstein, 1985) with 2000 replicates. We regarded tree topologies with bootstrap values (bs) of 70% or greater as sufficiently supported (Huelsenbeck and Hillis, 1993). For the Bayesian analysis, we considered posterior probabilities (bpp) of 95% or greater as significant support (Leaché and Reeder, 2002). Pairwise comparisons of uncorrected sequence divergences (p-distance) were calculated using MEGA4 (Tamura et al., 2007).

We compared coloration and body shape of *Pac. xanthospilos* and *Pac. changi* based on live specimens obtained by ourselves and ratios of body parts by use of data from Nishikawa et al. (2011a), where *Pac. xanthospilos* and *Pac. changi* were treated as locality sample 19 of *Pac. inexpectatus* and *Pachytriton* sp., respectively. Sample 19 in Nishikawa et al. (2011a) is clearly identified as *Pac. xanthospilos* because it was collected from the type locality of the species and has a large and robust body, and distinct dorsolateral orange dots, that characterize *Pac. xanthospilos* (Wu et al., 2012).

RESULTS

We obtained 1141 bp sequences of complete cyt b gene for 21 specimens including outgroups (Table 1). Of the 1141 nucleotide sites, 205 sites were parsimony informative (sequence statistics available upon request from the senior author). The likelihood values of the ML tree were -3918.365. The mean likelihood score of the Bayesian analyses for all trees sampled at stationarity was -3961.515.

Phylogenetic analyses employing two different optimality criteria (BI and ML) yielded nearly identical topologies. We therefore present only the ML tree in Fig. 1. Monophyly of *Pachytriton* was supported in both trees (bpp=95% and bs=84%). Within *Pachytriton*, four major clades were recognized: Clade I of *Pac. brevipes*, *Pac. changi*, *Pac. feii*, *Pac. granulosus*, and *Pac. xanthospilos*; Clades II of *Pac. archospotus*; Clade III of *Pac. inexpectatus*; Clade IV of *Pac. moi*. Clade I was further divided into four subclades: Subclade I-a of *Pac. xanthospilos* and *Pac. changi*; I-b of *Pac. brevipes*; I-c of *Pac. feii*; and I-d of *Pac. granulosus*. Besides Clade I and Subclade Ia, monophyly in each sample of *Pac. xanthospilos* (Samples 9–18) and *Pac. changi* (3 and 4) was supported in both trees.

Genetic distance between *Pac. xanthospilos* and *Pac. changi* (mean 2.9%, range 2.7–3.1%) was smaller than between any pair of the remaining congeners examined in this study (smallest: 5.3% [5.3–5.5%] in *Pac. brevipes* vs. *Pac. xanthospilos*; largest: 8.8% in *Pac. archospotus* vs. *Pac. inexpectatus*).

Our comparisons of coloration of *Pac. xanthospilos* and *Pac. changi* including topotypic specimens revealed the absence of tangible differences between them (Fig. 2). Dorsolateral orange dots have been reported as one of the diagnostic characteristics of *Pac. xanthospilos* (Wu et al., 2012), but the dots were also found in the type specimens of *Pac. changi*. Fine white dots, reported to be numerous on the dorsum and ventrum of the holotype of *Pac. changi* (Fig. 2E, F), were also found on the ventrum of topotypes of *Pac. xanthospilos*.

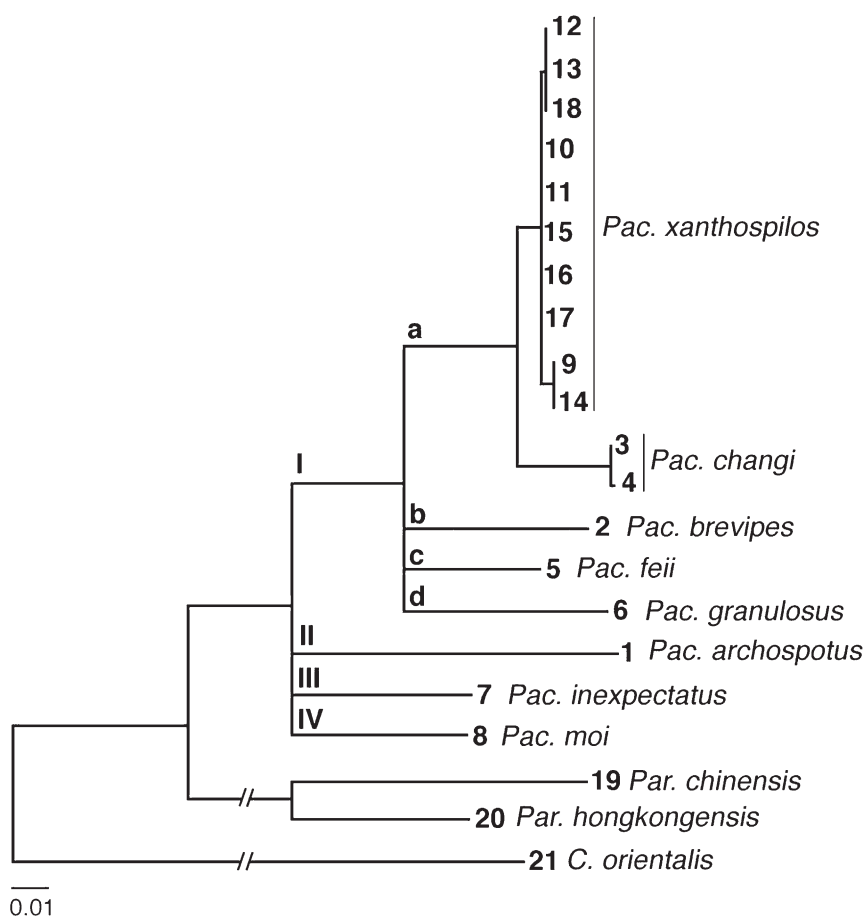


FIG. 1. ML tree based on the complete *cyt b* for *Pachytriton* species and outgroups (see Table 1). All bifurcated nodes are supported with BI-bpp \geq 95% and ML-bs \geq 70%, but polytomic nodes mean that relationship of lineages included are unsupported.

(Fig. 2B, D) although few in number. The pattern of ventral orange markings was variable among individuals and did not differ between the two species. In SVL and most of the character ratios examined, no clear difference was found between the two species (Table 2), although statistical tests could not be applied because of the small number of samples. *Pac. xanthospilos* tended to have a more robust body and larger values in tail width ratios than *Pac. changi*. Likewise in the shape of the upper tooth series, *Pac. xanthospilos* had smaller values in the ratio than *Pac. changi*.

DISCUSSION

As shown above, *Pac. xanthospilos* including topotypic specimens collected from the Yizhang, Hunan Province site (Samples 9–11 [type specimens], and 12–13), the Shaoguan, Guangdong Province site (14), and an unknown site (15–18) on Mt. Mang, and type specimens of *Pac. changi* from an unknown locality in China (3 and 4), formed a clade (Subclade I-a) with the smallest genetic distance between each pair of all nominal species compared (Fig. 1). Nishikawa et al. (2011a) showed that maximum intraspecific distances were 3.8% in *Pac. inexpectatus* and 3.3% in

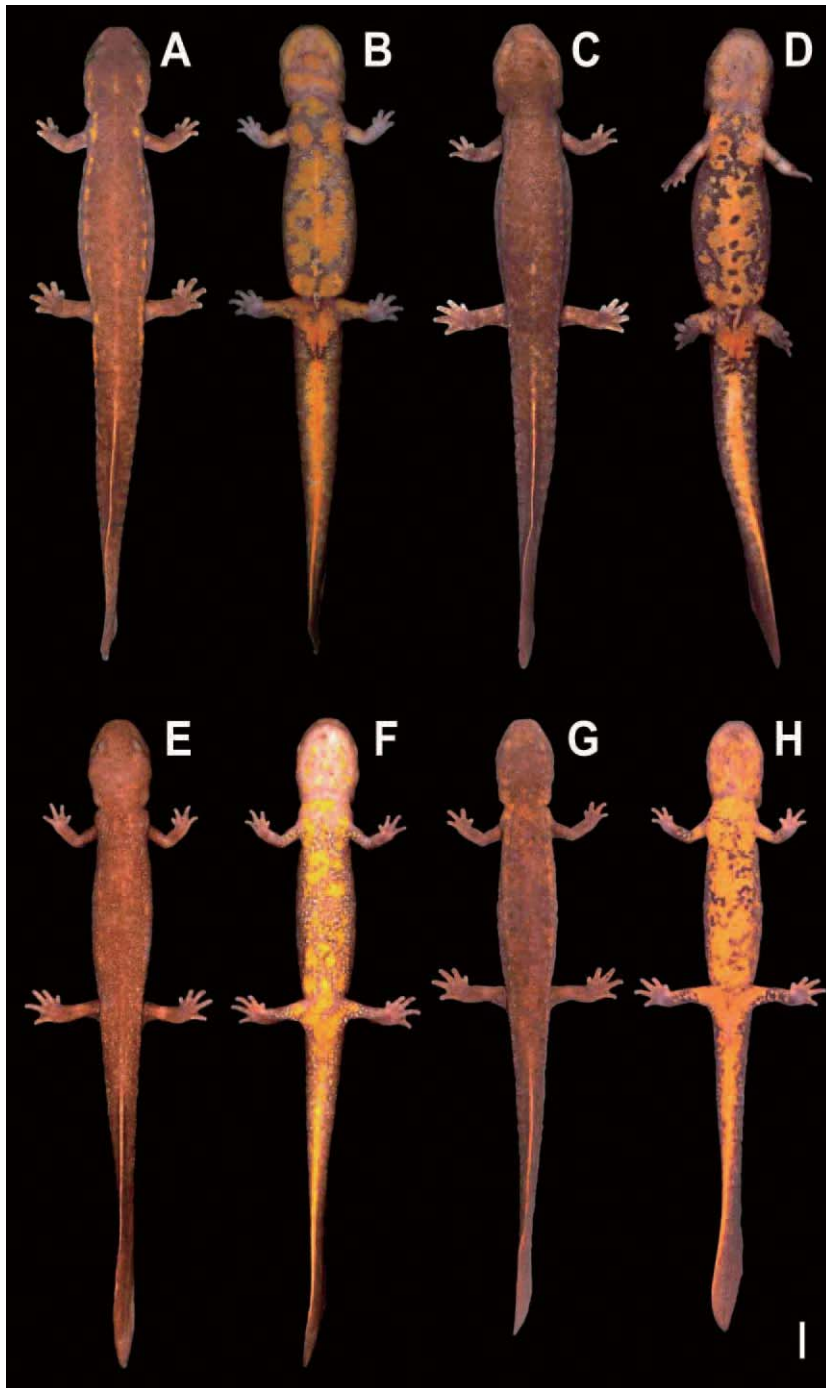


FIG. 2. Male topotype from Yizhang County, Hunan Province (CIB HN201108137: A and B) and male specimen from Shaoguan County, Guangdong Province (CIB GD201108042: C and D) of *Pachytriton xanthospilos* and male holotype (KUHE 39832: E and F) and paratype (KUHE 39763: G and H) of *Pachytriton changi*; dorsal (A, C, E, and G) and ventral views (B, D, F, and H). Scale bar=10 mm.

TABLE 2. Measurements of male specimens of *Pachytriton xanthospilos* and *Pac. changi* (means \pm SD of SVL (in mm) and medians of ratios of characters (R: %SVL) and VTL/VTW, with ranges in parentheses). Data were taken and modified from Nishikawa et al. (2011a). For character abbreviations, refer to Nishikawa et al. (2011a).

Types n	<i>Pac. xanthospilos</i>	<i>Pac. changi</i>			<i>Pac. xanthospilos</i>	<i>Pac. changi</i>	
	Topotypes 10	Holotype 1	Paratype 1		Topotypes 10	Holotype 1	Paratype 1
SVL	83.4 \pm 8.4 (67.9–96.3)	81.8	84.2	RTAL	99.8 (94.6–102.6)	101.1	105.1
RHL	24.9 (22.6–25.3)	25.4	25.2	RVL	6.4 (5.5–8.2)	6.1	5.7
RHW	19.3 (17.9–20.7)	18.2	18.8	RBTAW	13.2 (12.7–16.4)	12.2	12.0
RMXHW	21.1 (19.1–23.1)	19.3	20.5	RMTAW	10.5 (9.9–12.5)	8.3	7.5
RSL	9.4 (8.4–10.6)	9.8	10.1	RBTAH	12.2 (10.6–13.6)	10.8	10.0
RLJL	12.7 (12.1–14.6)	12.6	11.9	RMXTAH	16.2 (14.5–21.1)	15.0	15.4
RENL	7.3 (6.6–7.8)	7.3	7.2	RMTAH	13.8 (11.3–15.7)	14.7	15.2
RIND	6.9 (5.5–7.5)	6.8	7.1	RFLL	22.8 (20.7–24.0)	23.3	24.5
RIOD	8.0 (7.6–8.6)	7.0	7.7	RHLL	26.8 (24.4–28.9)	27.8	26.5
RUEW	2.4 (2.2–3.0)	2.9	2.4	RUJTW	8.7 (8.4–10.7)	10.6	11.2
RUEL	4.5 (3.8–5.2)	4.9	4.5	RUJTL	6.9 (5.7–7.3)	8.6	9.1
ROL	2.5 (2.0–2.8)	3.0	3.7	RVTW	5.8 (5.5–6.3)	5.6	6.2
RAGD	50.3 (45.5–53.2)	48.5	51.7	RVTL	10.4 (9.3–11.1)	11.0	12.1
RTRL	75.1 (74.7–77.4)	74.5	74.8	VTL/VTW	1.8 (1.5–2.0)	2.0	2.0

Pac. granulosus. The genetic distance between *Pac. xanthospilos* and *Pac. changi* (mean 2.9%, range 2.7–3.1%) obtained in the present study is not only smaller than the distances between species but also smaller than or equal to the ones within the species.

We also could not recognize any significant differences in coloration or body shape between these two species. *Pachytriton xan-*

thospilos tended to have a more robust body and wider tail than *Pac. changi*, but the body and tail thickness would be variable depending on the nutrition condition of the newts. Because the individuals of *Pac. changi* must have been transported from China to Japan and kept in captivity for a certain period before being sold at the pet shops, they might have become malnourished. Possible differ-

ences such as the shape of the upper tooth series need reexamination by adding more specimens, especially of *Pac. changi*, but as far as we can see, morphological differences between the two species are subtle and not comparable with clearer differences observed between the remaining congeners.

From these lines of genetic and morphological evidence, we conclude that *Pac. xanthospilos* Wu, Wang, et Hanken, 2012 (published on 13 July) is a subjective junior synonym of *Pac. changi* Nishikawa, Jiang, et Matsui, 2012 (published on 30 June) based on the principle of priority. Furthermore, the results of the present study indicate that the possibility of treating Mt. Mang population as a distinct subspecies of *Pac. changi* is low, because they differ only very slightly in DNA sequence and morphology. Even if the Mt. Mang population should prove to be a distinct subspecies in future studies, we still need to detect the exact type locality of *Pac. changi*, which will be the candidate for a nominotypical subspecies.

A recent taxonomic revision of *Pachytriton* was started by Shen et al. (2008), and followed by Nishikawa et al. (2009, 2011a, b) and Wu et al. (2012). In these studies, the number of species rapidly increased from two to eight, but the number has now been decreased to seven by the present results: *Pac. archospotus*, *Pac. brevipes*, *Pac. changi*, *Pac. feii*, *Pac. granulatus*, *Pac. inexpectatus*, and *Pac. moi*. Wu et al. (2012) basically followed Nishikawa et al. (2011b), who treated *Pachytriton labiatus* as a senior synonym of *Paramesotriton ermizhaoi* and transferred it to the latter genus. Wu et al. (2012), however, doubted that external and X-ray photographs of the lectotype specimen shown in Nishikawa et al. (2011b) were taken of different specimens. However, these photos were actually taken of the same lectotype specimen. The difference of postures in the two photographs simply resulted from the conditions in taking the X-ray photograph; the posture of limbs and tail in the X-rays were deformed intentionally using tape so as to show each skeletal element clearly and make the body fit on the X-ray film.

The present results clarified three localities where *Pac. changi* occurs. It is probable that *Pac. changi* is distributed in areas on the Nanling Mountain Range other than hitherto recorded, possibly in southern Hunan, north-eastern Guangxi, and northern Guangdong provinces. Individuals of *Pachytriton* resembling *Pac. changi* in appearance seem to occur in an area south of Mt. Mang, Guangdong Province (personal information from an anonymous collector to KN), and we need to explore unstudied areas to find more populations of the species. We need to keep conservation of the species in mind, because the size of each known population seems very small, and the habitats are always in danger of destruction by human activities. Furthermore, illegal collection for commercial purposes has been done even in the national parks.

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